Optimizing Carbon Dioxide and Light Levels during in Vitro Culture of *Theobroma cacao*

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Abstract. In vitro culture of axillary cotyledonary shoots of *Theobroma cacao* L. (cacao) under increasing CO₂ concentration from ambient to 24,000 ppm (culture tube levels) significantly increased total shoot elongation, number of leaves, leaf area per explant, and shoot dry and fresh weight. Although light was necessary for the CO₂ response, the effect of various photon fluxes was not significant for the measured growth parameters. Net photosynthesis estimated on the basis of CO₂ depletion in culture tubes increased 3.5 times from 463 to 2639 ppm CO₂, and increased 1.5 times from 2639 to 14,849 ppm CO₂, but declined from 14,849 to 24,015 ppm CO₂. Ethylene concentration in culture vessels increased under enriched CO₂ conditions. Depletion of nutrients (fructose, K, Ca, Mg, and P) from the medium was increased under enriched CO₂ conditions.

In conventional tissue culture, sugar is the carbon and energy source required for heterotrophic growth of the explant in vitro. Photosynthesis is partially limited by CO₂ depletion in sealed culture vessels under ambient concentration during the light period (Desjardins et al., 1988; Figueira et al., 1991, 1991a; Kozai, 1991). Elevated light combined with CO₂ enrichment has been proposed to stimulate photoautotrophism (Infante et al., 1989; Kozai, 1991). Explants cultured under autotrophic conditions have an improved survival rate during the acclimatization stage (Grout, 1988; Grout and Millan, 1985; Laforge et al., 1991; Lakso et al., 1986) and decreased contamination risks (Kozai, 1991). The CO₂ levels employed in photoautotrophic systems in vitro normally range from 1000 to 4500 ppm CO₂ (Desjardins et al., 1988; Infante et al., 1989; Kozai, 1991; Lakso et al., 1986).

The use of CO₂ enrichment for in vitro systems generally has been associated with the promotion of photosynthesis. However, high CO₂ concentration (chamber levels of 20,000 ppm) had a beneficial effect on shoot elongation and leaf development on detached axillary cotyledonary shoots and single-node cuttings from mature plants of *Theobroma cacao* L. (cacao), a species that had been considered recalcitrant under conventional protocols (Figueira et al., 1991). Woltering (1990) demonstrated that CO₂ concentrations above 10,000 ppm were beneficial to in vitro cultures of roses and gerbera, decreasing abscission, and senescence of leaves. These responses to such a high CO₂ concentration suggest that another process besides photosynthesis may be involved.

The primary objective of this study was to optimize the CO₂ concentration and light levels for in vitro growth and development of cacao axillary shoots and to investigate the physiological basis of the beneficial response to enriched CO₂. In addition, we hoped that these studies would help elucidate the cause of the recalcitrance of cacao under conventional in vitro culture.

Materials and Methods

In vitro axillary shoot proliferation from cotyledonary nodes. Open-pollinated pods from UF613 and from progenies of the crosses SCA6 x Catongo, CC42 x Catongo, CC42 x P-7, UF613 x SCA 6, Catongo x UF 676, and UF 676 x Catongo were obtained from the Intl. Cocoa Germplasm, Centro Agronomico de Investigacion y Enseñanza (CATIE), Turrialba, Costa Rica. Pods were washed, flame-sterilized, and opened in a laminar flow hood. The seeds were removed from the mucilaginous pulp and germinated on a Woody Plant Medium (WPM) (Lloyd and McCown, 1980) in baby food jars with 30 ml of medium supplemented with 88.8 mM fructose and 7 g·liter⁻¹ agar (Sigma). The medium was adjusted to pH 5.3 before autoclaving. The culture room was maintained at 26C under 16 h photoperiods. Epicotyls were removed 4 to 6 weeks after seeds had germinated to induce axillary shoots from the cotyledonary node (Janick and Whipkey, 1985), and the cotyledonary nodes were recultured on fresh medium. Axillary cotyledonary shoots were collected 4 to 6 weeks after decapitation.

Carbon dioxide chambers. Elevated CO₂ treatments were conducted in clear acrylic chambers located in a growth room. The chambers, not air tight, were continuously supplied with compressed CO₂ and air-mixed with a flowmeter (Matheson, East Rutherford, N.J.). The CO₂-air mixture was bubbled through distilled water and relative humidity inside the chambers ranged from 50% to 70%, the same as in the growth room. The CO₂ concentration in the chambers was measured using a gas chromatograph (model 8700; Carle GC, Broken Arrow, Okla.) with a thermal conductivity detector. Chambers were illuminated with VHO (SPELL) cool-white fluorescent lamps (General Electric, Pittsburgh). Chamber temperatures were higher than the growth room reaching 31C during the light period.

Optimization of light and CO₂ conditions. In the first experiment axillary cotyledonary shoots (≈5 cm) were transferred to tubes (44 ml) with 10 ml WPM supplemented with 88.8 mM fructose, previously demonstrated to be the optimum C source for cacao, and 7 g·liter⁻¹ agar (Sigma), with pH adjusted to 5.3 before autoclaving. The test tubes were capped with polypropylene closures (Magenta Corp., Chicago, Ill.) which permit gas exchange. The tubes were placed under one of six different CO₂ levels in the chambers [average CO₂ concentrations 463 ppm (growth room ambient), 2130 ppm, 5430 ppm, 9680 ppm, 19,750 ppm, and 30,200 ppm] and four photosynthetic photon fluxes (32,

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40, 51, and 106 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) obtained by cheese-cloth shading inside the chamber. Control test tubes (without axillary shoots) were included in all chambers. Carbon dioxide levels were monitored daily in chambers, and test tubes with and without shoots were sampled after 30 days in culture. There were 20 tubes in each CO_2 -light treatment. Data on total shoot elongation, number of leaves, and budbreak were taken 30 and 60 days after initiating treatment, and total leaf area was obtained after 60 days with a leaf-area meter (model LI-3000; LI-COR, Lincoln, Neb.). The parameters budbreak and total number of leaves were square root transformed.

Statistical analysis was performed using SAS (version 6.07) programs [analysis of variance (ANOVA) and regression]. The layout was a split-plot with no replications. Because no valid test existed for CO_2 and light levels, we used the variation among plants in every CO_2 -light combination for all statistical tests on the assumption that the error (a) and components (b) were 0.

In a second experiment to determine if light was necessary for the CO_2 effect, axillary cotyledonary shoots were placed in chambers with ambient or high CO_2 (20,000 ppm), under either high light (120 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) or in darkness achieved by loosely wrapping tube racks in aluminum foil. Each treatment consisted of 15 tubes. Data for leaf number and shoot elongation were obtained after 30 days.

Net photosynthesis estimation. Net photosynthesis estimation of tissue culture explants, using forced air-mixing systems and an infrared gas analyzer, causes conditions that differ from normal culture atmosphere (e.g., gas diffusion, altered water relations), restricting the validity of results (Kozai, 1991). Alternatively, Kozai and Iwanami (1988) proposed a method to estimate net photosynthesis based on differences in CO_2 concentration inside and outside the culture vessel: $P_n = -E \cdot v \cdot K_c \cdot (C_{in} - C_{out})$, where v is the headspace volume of culture vessel (44 ml); K_c is the conversion factor of CO_2 from volume to mass (1.83 $\text{mg}\cdot\text{cm}^{-3}$ at 25°C and 1 atm); and C_{in} and C_{out} are the CO_2 concentrations in ppm, inside and outside the vessel, respectively. The CO_2 concentrations were sampled daily for 12 days during the light period for tubes under six different chamber CO_2 levels (listed above) and at flux density of 106 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Tubes were sampled using a 1-ml syringe inserted through a silicone septa built into the lids. A different tube was evaluated each day to avoid a carry-over effect. The factor E is the number of natural air changes per hour of an empty vessel. E was estimated with carbon dioxide as a trace gas, using the following equation, proposed by Fujiwara et al. (1987): $E = -\ln[(K - K_{out})/(K_0 - K_{out})]/T$. K and K_0 are the CO_2 concentrations inside the vessel at time t and 0, respectively; K_{out} is the constant CO_2 concentration outside the vessel; and T is the time interval expressed in hours. This parameter E was estimated under our conditions to be equal to 1.03 air changes per hour.

Ethylene production under various CO_2 levels. In the first experiment, test tubes containing either 1, 2, or 3 axillary shoots cultured on 10 ml of WPM supplemented with 88.8 mM fructose and 7 g·liter⁻¹ agar, pH 5.3, were capped with air-tight rubber serum caps, and air was sampled for 72 h. There were five tubes per treatment. Ethylene was measured using a gas chromatograph (model 3000; Varian, Walnut Creek, Calif.) with an alumina column and flame-ionization detector. The second experiment was conducted using polycarbonate jars (Magenta GA7; Magenta Corp., Chicago) with a total volume of 375 ml, containing 50 ml of the same culture media described previously. Each lid had a silicone septa inserted. The jars were placed in chambers at 450 ppm (ambient), 2000 ppm, and 16,000 ppm CO_2 concentration. There were 10 jars per treatment. Ethylene was sampled at 1, 7, and 14 days after explantation.

Nutrient uptake. Axillary shoots were cultured in test tubes capped with plastic closures (Magenta Corp., Chicago) containing 10 ml WPM supplemented with 88.8 mM fructose and 7 g·liter⁻¹ agar, pH 5.3. Initial fresh weight of shoots was determined by weighing each test tube with medium, before and after explantation. Test tubes with and without axillary shoots were placed randomly under three CO_2 levels: ambient (423 ppm), 2250 ppm, or 30,970 ppm, all at PPFD of 90 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Tubes with medium but without shoots were used as a control. Axillary shoots that were sampled randomly at the beginning of the experiment, weighed, and frozen represent the initial nutrient measurements. Every 10 days for 60 days, five test tubes were sampled for each CO_2 treatment. The shoots were removed from the tubes, weighed, and stored frozen. The five control tubes without shoots also were harvested and stored. At the end of the experiment, shoots (leaves plus stems) and the media were dried at 60°C for 24 h and dry weights were determined. The experiment consisted of 190 tubes (three CO_2 treatments $\times$ six collection dates $\times$ five tubes with and five tubes without shoots, plus ten tubes sampled at time 0). The media depletion experiment was based on three plants from each CO_2 chamber at each sampling date.

Dried axillary shoots (stem and leaves) were ground with a mortar and pestle, and a sample of 100 mg dry weight for each CO_2 treatment at each 10-day interval was digested using a perchloric acid-hydrogen peroxide method (Adler and Wilcox, 1985). A 100-mg sample of dried medium also was digested under the same conditions. Potassium, Ca, and Mg contents were determined using an atomic absorption spectrophotometer (model Spectra AA 10; Varian Techtron Pty. Ltd., Mulgrave, Victoria, Australia). Phosphorus was determined by the molybdenum blue method (ammonium molybdate-sodium sulfite, 1,2,4-naphthol sulfonic acid procedure) (Jackson, 1948). Fructose was determined enzymatically with a kit supplied by Boehringer-Mannheim Biochemicals (Indianapolis).

Results

Effect of CO_2 concentration and illumination

The CO_2 effect after 60 days was highly significant (1% level) for total shoot elongation, total number of leaves, and leaf area per explant, with the highest values at the highest CO_2 level (30,200 ppm) (Table 1 and Fig. 1). In contrast, budbreak decreased with increasing CO_2 concentrations (significant at $P=0.05$). When total CO_2 effect variance was partitioned into components, the linear component was highly significant for total elongation and total leaf area (Table 1). The total number of leaves was significant for the linear and quadratic component. The total and partitioned light components were not significant for any of the measured growth parameters. The interaction between CO_2 and light was not significant for total shoot elongation or total leaf area. Our analyses using plant-to-plant error assumed error (a) and error (b) variance components equalled 0. That this assumption was reasonable was evident in that the mean squares for the CO_2 quadratic and higher order sources for stem elongation and leaf area were less than the plant-to-plant error as shown in Table 1. In the case of number of leaves and number of budbreaks, the cubic and higher order sources were either less or nonsignificant from plant-to-plant error.

In the second experiment, axillary cotyledonary shoots were placed under high or low CO_2 at high light or dark conditions to determine if the CO_2 effect could be separated from photosynthesis per se. At high light, CO_2 enrichment increased the number of leaves and shoot elongation, but in darkness the number of leaves

Table 1. Main effect of increasing light and CO₂ levels on axillary cotyledonary shoot of cacao elongation, number of leaves, leaf area, budbreak, and root number, 60 days after treatments were imposed.

CO ₂ (ppm)	Light (μmol·m ⁻² ·s ⁻¹)	Total elongation (mm)	No. of leaves/ explant	Leaf area (cm ²)	Budbreak/ explant
463		9.7	2.4	4.0	1.2
2130		13.2	2.8	5.1	1.0
5430		13.6	3.8	6.6	0.8
9680		14.0	3.6	7.8	0.8
19,750		16.2	4.6	8.8	0.6
30,200		18.1	4.9	11.7	0.6
Significance		**	**	**	*
Linear		**	**	**	**
	32	14.3	3.8	6.5	0.8
	40	14.8	3.6	7.0	1.0
	51	15.0	3.5	7.8	0.6
	106	12.4	3.4	7.9	0.8
	NS	NS	NS	NS	NS
<i>Analysis of variance</i>					
Mean squares					
Source of variation	df	Stem elongation	No. of leaves ^z	Leaf area	No. budbreak ^z
CO ₂	5	624.4**	6.34**	604.2**	1.51*
CO ₂ linear	1	2730.4**	25.18**	2866.3**	4.34**
CO ₂ quadratic	1	68.8	5.79**	34.0	2.85*
Light	3	125.8	0.21	49.3	0.90
CO ₂ × light	15	92.8	0.60	41.8	1.12*
Error	426	139.7	0.57	36.5	0.54

*,** Nonsignificant or significant at $P = 0.05$ and 0.01 , respectively.
^zSquare root transformed.

Fig. 1. Response of cacao cotyledonary axillary shoots to CO₂ concentrations.

Table 2. Effect of CO₂ and light on shoot elongation and leaf development of axillary cotyledonary shoots of cacao.

PPF (μmol·m ⁻² ·s ⁻¹)	CO ₂ (ppm)	
	500	20,000
<i>Shoot elongation (mm ± SE)</i>		
0	3.9 ± 2.2	1.8 ± 0.6
135	1.9 ± 0.5	6.1 ± 2.1
<i>No. of leaves ± SE</i>		
0	0.6 ± 0.2	0.5 ± 0.2
135	1.4 ± 0.3	2.8 ± 0.3

decreased at both CO₂ levels, whereas shoot elongation decreased at high CO₂ (Table 2). The increase in elongation at low CO₂ in darkness appears to be an etiolation effect.

Net photosynthesis estimation

Estimates of net photosynthesis using the CO₂ concentration differential from inside tubes with shoots to chamber concentration showed an increase in net photosynthesis that was proportional to CO₂ up to 30,200 ppm (Table 3). This appears to be an overestimation of net photosynthesis because CO₂ concentrations inside tubes without shoots were much lower than chamber levels at the two highest CO₂ concentrations. Estimation of net photosynthesis using the CO₂ differential within tubes with and without shoots indicated a 3.5-fold increase from 463 to 2639 ppm CO₂, and a 1.5-fold increase from 2639 to 14,849 ppm CO₂ (Table 3). Net photosynthesis estimates declined, however, from 14,849 to 24,015 ppm CO₂.

Ethylene production by cacao shoots

In the first experiment, cacao shoots were cultured in test tubes sealed with air-tight serum closures. The presence of cacao shoots (one to three) in the tube increased the levels of measured ethylene over time (Table 4), as compared to the control (no shoots). Ethylene concentration, however, also increased in tubes without shoots. (This increase in ethylene in tubes with media but without shoots has been found in other experiments.) Ethylene concentrations in tubes with one to three shoots were similar until 4 h after explantation. From 4 to 22 h, ethylene concentration increased sharply in tubes with two or three shoots. The peak for ethylene detection occurred between 24 and 48 h. Ethylene concentration at 48 h was directly proportional to the number of shoots present, suggesting that large amounts of ethylene produced by cacao axillary shoots were retained in the tube. It should be noted that conventional culture conditions employ gas-permeable closures.

Ethylene levels were determined in Magenta GA7 vessels (not air-tight) under three CO₂ regimes, each containing one shoot. Elevated CO₂ concentration increased ethylene concentration (Table 5). The ethylene concentration at 2000 ppm was significantly higher than at ambient, and at 16,000 ppm was significantly higher than at either ambient or 2000 ppm. Thus, high CO₂ concentration led to an increase of detectable ethylene. Ethylene concentration was maximal at 7 days.

Nutrient uptake

Shoot growth. Because initial fresh weight significantly affected the growth of the shoots for all treatments (covariate analysis, data not shown), shoot growth data have been expressed as cumulative increases in fresh weight (difference of fresh weight between a given time and the beginning of the experiment) (Fig. 2),

Table 3. Estimation of net photosynthesis at various CO₂ levels, under PPF of 104 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The number of air changes per hour was considered constant for all CO₂ levels ($E = 1.03\cdot\text{h}^{-1}$).

Chamber (ppm) [a]	CO ₂ concn (ppm)		Net photosynthesis ($\mu\text{g CO}_2/\text{plantlet per h}$)	
	Tube- shoot [b]	Tube + shoot [c]	Difference between tube with shoot to chamber [(a-c) $\times$ constant]	Difference between tube with shoot to tube without shoot [(b-c) $\times$ constant]
463 $\pm$ 8 ^z	409 $\pm$ 55	235 $\pm$ 51	26.5 $\pm$ 4.3	20.5 $\pm$ 3.9
2,130 $\pm$ 85	2639 $\pm$ 356	2,383 $\pm$ 510	72.2 $\pm$ 17.0	73.2 $\pm$ 17.1
5,430 $\pm$ 258	5469 $\pm$ 431	5,101 $\pm$ 380	83.5 $\pm$ 30.3	77.6 $\pm$ 26.7
9,680 $\pm$ 341	9965 $\pm$ 698	9,439 $\pm$ 632	118.7 $\pm$ 41.0	90.0 $\pm$ 14.8
19,750 $\pm$ 524	14,849 $\pm$ 543	13,856 $\pm$ 610	395.8 $\pm$ 43.5	110.9 $\pm$ 27.4
30,200 $\pm$ 855	24,015 $\pm$ 716	23,663 $\pm$ 762	474.9 $\pm$ 54.1	76.7 $\pm$ 18.9

^z $\pm$ SE.

Table 4. Determination of ethylene accumulation from cacao axillary cotyledonary shoots cultures in test tubes capped with air-tight serum cap.

Time (h)	Ethylene concentration (nl-liter ⁻¹)			
	No. shoot			
	0	1	2	3
0.5	14.2 $\pm$ 0.6 ^z	25.4 $\pm$ 2.5	17.8 $\pm$ 1.2	16.6 $\pm$ 0.0
1.5	14.2 $\pm$ 0.4	26.6 $\pm$ 0.9	19.1 $\pm$ 1.2	16.6 $\pm$ 0.0
4	16.6 $\pm$ 0.0	30.8 $\pm$ 3.1	37.6 $\pm$ 4.9	30.6 $\pm$ 0.9
22	22.8 $\pm$ 1.3	43.8 $\pm$ 5.4	140.8 $\pm$ 44.1	100.0 $\pm$ 29.6
24	22.8 $\pm$ 0.4	45.5 $\pm$ 4.6	146.3 $\pm$ 45.2	109.2 $\pm$ 29.6
48	31.5 $\pm$ 1.9	63.0 $\pm$ 12.3	120.4 $\pm$ 14.8	153.7 $\pm$ 50.0
72	21.6 $\pm$ 1.6	48.6 $\pm$ 10.4	96.9 $\pm$ 27.7	86.1 $\pm$ 36.1

^z $\pm$ SE.

Table 5. Ethylene concentration detected on cacao axillary shoots cultures in polycarbonate Magenta GA7 vessels, closed with polypropylene lids, at various times after explantation.

Time (days)	Ethylene (nl-liter ⁻¹)			
	450 ppm CO ₂	2,000 ppm CO ₂	16,000 ppm CO ₂	Avg
1	3.2 $\pm$ 0.4 ^z	3.2 $\pm$ 0.4	7.3 $\pm$ 2.0	4.6
7	6.8 $\pm$ 0.4	17.1 $\pm$ 2.3	22.9 $\pm$ 1.1	15.6
14	7.3 $\pm$ 2.0	7.4 $\pm$ 1.1	12.6 $\pm$ 1.3	9.2
Avg	5.8 a ^y	9.1 b	14.1 c	

^z $\pm$ SE.

^y Mean separation by Duncan's multiple range test at $P = 0.05$.

and as fresh weight gain per day (Fig. 3). Although dry weight increases followed fresh weight fairly closely, only fresh weight data are discussed since initial dry weight of shoots in treatment could not be assessed. Final fresh weight of shoots was significantly improved by increasing CO₂ (Fig. 2). Cumulative increase in fresh weight initially was minor, but after 30 days the differences between ambient and CO₂ enrichment treatments enlarged, but only with small differences between the two elevated CO₂ regimes (Fig. 2). Shoots kept at ambient CO₂ had little increase in fresh weight for 50 days (while dry weight actually decreased during this period).

Increasing CO₂ concentration significantly increased the growth rate as determined by the fresh weight, with the highest rate occurring at the highest CO₂ level (Fig. 3). The fresh weight growth rate generally was constant over time. The improved growth under increased CO₂ concentration was a result of cumulative increase over time. Although the average increase in fresh weight from

Fig. 2. Fresh weight accumulation by axillary cotyledonary shoots of cacao grown under three CO₂ regimes.

Fig. 3. Daily fresh weight increase in cotyledonary axillary shoots of cacao grown under three CO₂ regimes.

Fig. 4. Fructose depletion from media with cotyledonary axillary shoots of cacao grown under three CO₂ regimes.

shoots cultured under CO₂ concentrations of 2250 and 30,970 ppm did not differ significantly, the fresh weights at the very high CO₂ levels always were highest.

Fructose. Fructose was depleted from the medium in all CO₂ treatments, but at different rates (Fig. 4). Under both elevated CO₂ conditions (2250 and 30,970 ppm), fructose was consumed at similar rates, with about 40% of the initial concentration used during the first 30 days, and about 70% for the whole 60-day period. However, at ambient CO₂ only 5% of the original concentration was consumed during the first 30 days, and about 50% in the following 30-day period. The consumption of fructose paralleled the net cumulative growth response.

Elements. The initial fresh weight of explants did not affect the rate of nutrient uptake for any of the elements analyzed (covariate analysis, data not shown). The three CO₂ levels tested caused no striking differences in the nutrient concentration of shoots (leaves plus stems), and these results were consistent with the values reported for field-grown plants by Murray (1967) (Fig. 5).

Significance of the CO₂ effect based on analysis of variance (ANOVA) is shown in Table 6. In the nutrient depletion experiment K and Ca showed a significant CO₂ × date interaction, so interaction mean square was used to compute significance for main effects, as this was the more conservative test. Potassium depletion of the medium was proportional to shoot growth, i.e., depletion was greatest under enriched CO₂ (Fig. 5A). Nutrient depletion from the medium under ambient CO₂ was significantly lower than under enriched CO₂, but there was no apparent difference in nutrient depletion between both enriched CO₂ treatments after 20 days. At the end of the 60-day period, about 50% of the initial K remained in the medium under ambient conditions, as compared to 20% and 24%, respectively, under 2250 and 30,970 ppm CO₂. Potassium concentration of shoots under ambient CO₂ increased over time (Fig. 5B). Potassium tissue concentration from 30 to 60 days was highest under ambient CO₂.

The rate of Ca depletion from the medium was less than that of K depletion and was not associated with shoot growth (Fig. 5C). After 60 days, the remaining Ca level was about 77% for ambient and about 70% for enriched CO₂ conditions. The CO₂ effect was not significant, but under the ambient CO₂ regime Ca in the medium usually remained at higher concentration than under

Fig. 5. Media depletion (left) and tissue concentration (right) of cotyledonary axillary shoots of cacao grown under three CO₂ regimes (423, 2250, or 30,970 ppm): potassium (A and B); calcium (C and D); magnesium (E and F); and phosphorus (G and H).

Table 6. Significance of CO₂ and time effect on nutrient depletion by in vitro-grown axillary shoots.

Source of variation	DF	K	Ca	Mg	P
CO ₂	2	*	NS	NS	*
Linear	1	NS	NS	NS	NS
Quadratic	1	**	NS	*	*
Time	6	**	NS	**	**
Linear	1	**	**	**	**
Quadratic	1	NS	NS	*	NS
CO ₂ × time	12	*	**	NS	NS

NS, *, ** Nonsignificant or significant at $P = 0.05$ or 0.01 , respectively.

enriched CO₂ (Fig. 5C). There was no strong evidence that Ca concentration in shoots varied due to CO₂ treatment, but concentration was generally higher under ambient CO₂ (Fig. 5D).

Magnesium was depleted from the medium at about the same rate for all CO₂ treatments, implying that the rate of Mg uptake was unaffected by growth (Fig. 5E). After 60 days, Mg levels in medium ranged from about 62% for ambient CO₂, 53% at 2250 ppm CO₂, and 65% at 30,970 ppm CO₂. Under enriched CO₂ treatments, Mg concentration of shoots tended to decline over time (Fig. 5F), but under ambient CO₂ concentrations remained constant after an initial drop.

The rate of P depletion from the medium was significantly affected by CO₂ and was proportional to shoot growth (Fig 5G). At the end of 60 days, P was essentially depleted from all CO₂ treatments, but depletion was least under ambient CO₂. About 13% of the original P remained at 2250 ppm CO₂, as compared to 21% at 30,970, and 32% at ambient (423 ppm CO₂). Tissue P levels were highest in ambient CO₂ (Fig. 5H).

Discussion

The results of these experiments confirm the recalcitrance of cacao under conventional protocols and the promotive effects of CO₂ enrichment reported by Figueira et al. (1991). Shoot growth and development improved as CO₂ concentration in the chambers increased from ambient to 30,200 ppm (tube concentration = 24,015 ppm) but the effect was not directly proportional, as concentration was greatest at the lower levels of increase (Fig. 1). The most direct developmental response to increased CO₂ was increased leaf area (Fig. 1C) attributable both to increased number of leaves and increased leaf surface. Another nonphotosynthetic effect of increasing CO₂ was decreased budbreak.

The simplest explanation for the CO₂ effect on in vitro performance is increased photosynthesis and, indeed, light was necessary for the CO₂ effect (Table 2). The increase in dry weight of plants grown under enriched CO₂ concentration (Fig. 3) is typically a result of increased C fixation (Mott, 1990). However, the effect of light levels was not significant (Table 1), suggesting that although photosynthesis plays a key role in improving cacao shoot growth and development, an additional effect is involved. Estimation of net photosynthesis based on CO₂ depletion within tubes indicated an increase up to 14,849 ppm but a decline at 23,663 (Table 3), despite a corresponding increase in growth and development.

The full explanation for the response of cacao in vitro to such high levels of CO₂ remains baffling. Guers (1985) reported that net photosynthesis of cacao cuttings in the field was nearly saturated at 600 ppm CO₂. Increased net photosynthesis may derive either from improved CO₂ uptake, inhibition of photorespiration, or both. Carbon dioxide uptake may be lower under in vitro conditions due to the absence of turbulence, which contributes to a large boundary layer resistance. Carbon dioxide effects on stomatal regulation are unlikely to be involved because of the high humidity in vitro environment. An unexplored explanation was alteration of intracellular pH (Bown 1985).

We considered the possibility of a CO₂ effect on ethylene that could suppress growth, but, in fact, ethylene concentration increased with increasing CO₂ concentration (Table 4 and 5). It is possible that CO₂ could be reducing ethylene response while stimulating its production (Sisler and Wood, 1988).

Elevated CO₂ could conceivably influence nutrient absorption. Cacao shoots contain large amounts of polysaccharides (Brooks and Guard, 1952), which have been associated with recalcitrance

in vitro (Blake and Maxwell, 1984; Legend et al., 1986; Orchard et al., 1979; and Passey and Jones, 1983). Gum exudation from the cut surfaces of shoots might interfere with in vitro nutrient uptake and/or availability (Figueira et al., 1989). Cacao gum is acidic, rich in uronic acids, and negatively charged under pH of 5.3. Large amounts of bound Ca were detected in gum isolated from liquid culture of cacao shoots (Figueira et al., 1991b). Shoots under conventional in vitro culture perform better if roots are present (Figueira et al., 1991).

Fructose uptake, measured by medium depletion, was lowest under ambient CO₂ levels (Fig. 4). Similarly, under ambient CO₂, nutrient uptake as measured by medium depletion tended to be lowest, while nutrient content of tissues generally was highest, indicating that nutrients were being absorbed but were underutilized under low CO₂. The nutrient data, however, produced no clear evidence that elevated CO₂ overcomes any interference by gum on nutrient uptake. The low absorption of Ca and Mg was not improved by CO₂ enrichments despite improved growth. Thus, it is unclear if the increased nutrient uptake found under enriched CO₂ atmospheres was the result or the cause of increased growth. The fact that nutrient uptake occurs without significant growth at ambient CO₂ levels suggests that the stimulatory effect of CO₂ is not a direct effect of CO₂ to increase uptake, but rather a direct effect on growth itself.

We conclude that in vitro growth and development of cacao is promoted by increasing levels of CO₂ up to tube concentrations of 24,015 ppm. Part, but not all, of the response to high CO₂ appears to be explained by an increase in net photosynthesis. The assumption that stem gums are the cause of recalcitrance could not be confirmed or disproved on the basis of nutrient uptake under different CO₂ concentrations. Finally, we suggest that CO₂ enrichment with tube levels as high or higher than 20,000 ppm might be beneficial for in vitro culture of other recalcitrant tropical species.

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